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ENHANCEMENT OF AMYLASE STABILITY BY IMMOBILIZATION DURING SIMULTANEOUS COLD HYDROLYSIS AND FERMENTATION OF WHEAT STARCH GRANULES

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Immobilized Barley α-Amylase for Hydrolysis of Starch Particles

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A. Executive Summary

Liquid fuel is a very precious resource used abundantly and somewhat indiscriminately by modern man. The primary source of liquid fuel is currently crude oil, which is becoming harder and more expensive to recover as conventional reserves are depleted and as foreign suppliers increase the price for their declining reserves. With the wide majority of atmospheric scientists now agreeing that global warming is already well underway, there are new incentives to replace crude oil as our liquid fuel source in order to reduce the build-up of greenhouse gases in the environment. Finally, the historic trend for the monetary value of Saskatchewan wheat has been on the decline since the early part of the twentieth century and with huge subsidies provided by foreign governments to their wheat farmers, it appears this general trend will continue.

For the three reasons noted above: 1) rising costs of crude oil, 2) global warming and 3) declining value of wheat, it is clear that the Province of Saskatchewan (supplier of half of Canada's wheat) has a strong incentive to become a major player in the development of the Canadian bioethanol industry, by developing new technologies to convert wheat (or other Saskatchewan agricultural materials) to ethanol. Saskatchewan Agriculture and Food is the main supporter of these developments, and has provided the financial support that has resulted in this academic investigation.

Previous research by the authors (see final reports under SADF contracts 94000287, 1996; 96000093, 1998; and journal papers attached to this report) has shown that barley α-amylase is the best enzyme to use to carry out cold wheat starch granule hydrolysis. This cold reaction reduces the need to cook wheat starch (or any other starch

material) in the bioethanol industry thereby greatly reducing the cost of producing bioethanol. In fact, the successful scale-up of this new technology was reported to result in a bioethanol plant that would have a pay-back period of only two years and would provide a discounted rate of return of 46 % (see Lang et al, Energy Sources, 2001).

Although barley α-amylase is relatively inexpensive, in previous studies it was found to degrade after 100 hours of use. In this work, immobilization of barley α-amylase was investigated as a means to stabilize this biochemical. For the first time, barley α-amylase has been successfully immobilized on inexpensive carrier particles, silica sand. An optimum procedure has been developed which fixes the enzyme molecules and results in high activity particles. Furthermore, it was proven that this immobilized enzyme could hydrolyze starch granules to sugars at rates that were similar to those achieved with soluble barley α-amylase, and at starch concentrations as high as those achieved with soluble barley α-amylase.

The immobilized barley α-amylase was shown to have thermostability three times higher than the soluble enzyme molecules. However, when used in a fed-batch operational mode, where starch particles were continuously being hydrolyzed by the same immobilized enzyme particles, the immobilized enzyme decayed at the same rate as observed earlier with soluble barley α-amylase. This means that the immobilization process developed here did not result in extending the usage of barley α-amylase for use in wheat starch hydrolysis.

Successful models of the hydrolysis process, inhibition and decay of the immobilized barley α-amylase were developed and shown to fit the experimental data well. The models not only help to explain the magnitudes of improvements in thermostability of

the immobilized enzyme compared to the soluble enzyme, but also show that the operational instability of the immobilized enzyme is four times higher than its thermal instability and this is identical to the operational instability of soluble barley α-amylase.

At present, it would not be of value to scale-up an immobilized barley α-amylase process. The previous design developed by the authors, using an ultrafiltration membrane in order to recycle the barley α-amylase, is a superior method to produce bioethanol. Nevertheless, further research support should be considered to develop novel environmental (temperature or pH) or chemical ways to stabilize immobilized barley α-amylase. It could be true that simple conditioning procedures of the active enzyme particles right after they have been formed or during the immobilization procedure, could produce a more stabilized product which would then be of great value to the bioethanol industry. For instance at a 10 million litre per year plant, a stable amylase enzyme could save the bioethanol process $500K per year.

At the very least, this work has proven that immobilization can be successfully achieved with barley α-amylase and that the immobilized enzyme can effectively hydrolyze starch granules.

B. Abstract

Barley α-amylase has been immobilized on fine silica particles with diameters between 0.5 and 10 μm using a covalent binding method. Immobilization procedures were adjusted to optimize enzyme activity. The effects of product inhibition, thermal stability and operational stability have been determined. The feasibility of using the immobilized enzyme to hydrolyze wheat starch particles at temperatures below the gelatinization temperature (< 55 °C) was proven. The optimal conditions for the hydrolysis were found to be: pH 4.5, 40 °C, calcium ion concentration 0.002 M and immobilized enzyme loading of 30 mg/ml. At this condition, the immobilized enzyme was able to hydrolyze wheat starch particles at concentrations as high as 100 mg/ml with a final conversion of 90 % after 24 hours of operation. Maltose and glucose were found to inhibit the immobilized enzyme in a similar manner as reported previously using soluble enzyme. Although the thermal stability of the immobilized enzyme was superior to the soluble enzyme, the immobilized enzyme degraded at the same rate as the soluble enzyme during cold wheat starch hydrolysis (operational stability unchanged). Model equations are presented for product inhibition, hydrolysis kinetics and enzyme decay. Using best-fit parameters, the equations are shown to fit the experimental data well.

Keywords: immobilized enzyme, wheat starch, inhibition, degradation, stability, models

C. Technical Report

Introduction and background

The production of ethanol from starch consists of two steps: hydrolysis of starch molecules and fermentation of the hydrolysis products. Hydrolysis of starch can be achieved using either acid or enzyme catalysts (Schenck and Hebeda, 1992). Conventional enzyme hydrolysis involves liquefaction and saccharification of starch molecules, both of which are normally carried out above 90 °C to precook and dissolve the particles. Precooking requires a large amount of thermal energy, consisting of 30 to 40 % of all energy spent for alcohol production (Mikuni *et al.* 1987). In the 1980s, ethanol fermentation of starches at temperatures below the liquefaction and saccharification temperatures had rarely been investigated (Mikuni *et al.* 1987). Textor *et al.* (1998) studied the possibility of carrying out wheat starch hydrolysis using soluble α-amylases at temperatures below the gelatinization temperature. Barley α-amylase was found to be the most suitable enzyme for liquefaction when used at pH 4.5 and 45 °C. Both Motesinos and Navarro (2000) and Lang et al. (2001a, 2001b) studied the simultaneous, cold hydrolysis of starch particles and fermentation to produce ethanol from wheat flour using soluble enzymes and yeast. One of the major problems was the significant rate of enzyme deactivation. It was reported that at 45 °C, soluble barley α-amylase deactivates at a rate of 1 % per hour (Hill *et al.* 1997).

An attempt has therefore been made to stabilize barley α-amylase for use in the cold wheat starch hydrolysis process. Stabilization of the enzyme would improve economics, reducing the need to purchase new enzymes and hence reducing the cost of ethanol

production as a whole. One of the most effective ways to achieve stabilization is by the use of immobilized enzyme (Bailey and Ollis, 1986). Multipoint covalent attachment to solid supports has been used to stabilize several industrial enzymes (Guisán et al., 1992). The formation of the rigid enzyme - support linkage provides great kinetic and thermodynamic stabilization of the three dimensional structure of the active catalytic site. The immobilized enzyme molecules may also be stabilized against denaturing agents that promote unfolding processes that can destroy the active site (Mozhaev, 1992).

Kvesitadze and Dvali (1982) used a covalent cross-linking method coupled with glutardehyde or titanium tetrachloride to fix mold and bacterial amylase to silica carriers at pH 4.8. The presence of both calcium chloride at 0.05 M and ethanol at 12 % (w/v) were found to improve enzyme stability. Storage activity was retained for more than six months when the immobilized enzyme was kept in 0.015 M acetate buffer at pH 4.8 and 4 ^{o}C. Immobilization of α-amylase on ultra-fine silica particles was performed by Kondo *et al.* (1994) using both physical adsorption and covalent cross-linking (using glutaraldehyde as the cross-linking reagent). Both methods provided similar retained activities. The covalently bound enzyme was then used for hydrolysis studies and it was reported that the immobilized enzyme could be reused with its activity retained. De Cordt et al. (1992) reported that the thermostability of *Bacillus licheniformis* α-amylase was improved after the enzyme was immobilized on silane coated silica carriers. Roig et al. (1993) studied α-amylase immobilized on plastic supports. The presence of $CaCl_2$ was found to be important for storage stability of the immobilized enzyme.

In this study, an optimum technique for immobilizing barley α-amylase on silica particles has been developed. Product inhibition, thermal degradation and operational stability kinetics during cold starch particle hydrolysis were then determined. Finally, mathematical models for inhibition, degradation and immobilized hydrolysis are reported.

Mathematical model

The kinetics of enzymes in solution acting on insoluble substrates has been reviewed by Bailey and Ollis (1986). The enzyme molecules attach to the surface of the substrate granules and catalyze the transformation of the substrate molecules. In this situation, the reaction rate will reach a limit as the enzyme concentration is increased. To develop a model, the normal roles of substrate and enzyme are reversed and the enzyme molecules are assumed to adsorb onto the surface of the substrate. Textor et al. (1998) studied cold wheat starch hydrolysis using soluble barley α-amylase and found the Bailey and Ollis (1986) model fit starch particle hydrolysis well. With immobilized enzyme, the situation becomes more complex, since both the enzyme and substrate molecules are insoluble and the enzyme particles are faced with an extra complication of coming in contact with the substrates particles. However, this mass transfer problem can be circumvented if the diameters of the immobilized enzyme particles are small so that there is no increased difficulty to attach to the substrate molecules. The starch granules used in this study had a mean diameter of 21 μm while silica particles used for enzyme immobilization has a diameter range of 0.5 to 10 μm. The kinetic model for soluble enzyme and insoluble

substrate was found to fit the experimental hydrolysis data obtained for the immobilized enzyme well.

The equilibrium reaction model as proposed by Bailey and Ollis (1986) is:

$$E + A \underset{Kdes}{\overset{Kads}{\rightleftarrows}} EA \xrightarrow{k3} E + P \qquad (1)$$

where E represents the free enzyme at any time, A is a vacant site on substrate surface, EA is the occupied site on the substrate surface, P is the product, k_{ads} represents the rate constant for adsorption of enzyme, k_{des} is rate constant for desorption of enzyme and k_3 is the rate constant for the hydrolysis reaction. Textor et al. (1998) developed the following differential model which predicts the disappearance of starch particles as a function of time:

$$\frac{dS}{dt} = \frac{-KSE_0}{K_A + E_0} \qquad (2)$$

Taking $P = S_0 - S$, where P is the hydrolysis product, Equation 2 becomes:

$$\frac{dP}{dt} = \frac{K(S_0 - P)E_0}{K_A + E_0} \qquad (3)$$

The term thermostability depicts the enzyme deactivation without the presence of the actual substrate (wheat starch, in this case) while operational stability accounts for the presence of substrate. Even though experimental data obtained from a thermostability study cannot be used directly in a reaction kinetics analysis, the study does serve to cast some useful insight into the behavior of the immobilized enzyme with a change in temperature. In the simplest model, active enzyme molecules are hypothesized to undergo irreversible structural or chemical change to an inactive form at a rate proportional to the active enzyme concentration:

$$\frac{dE}{dt} = -k_d E \qquad (4)$$

k_d is the first-order, enzyme specific deactivation rate constant. k_d depends on the temperature and can often be described by the Arrhenius equation (Bailey and Ollis, 1986). At constant environmental conditions, the integrated form of Equation 4 gives:

$$\frac{E}{E_0} = \exp(-k_d t) \qquad (5)$$

Kvesitadge and Dvali (1982) reported first order deactivation kinetics for α-amylase immobilized on silica particles. Rollings and Thompson (1984) found a similar deactivation pattern for soluble α-amylase. Roig et al. (1993) proposed identical deactivation kinetics for α-amylase immobilized on plastic supports.

Frequently, a pure, simple first-order model as described above is sufficient to adequately represent enzyme deactivation kinetics. Equations 4 implies that degradation of the enzyme is not a function of time. Houng et al. (1992) proposed a time-dependent enzyme deactivation model for amylases. Their model predicted a quadratic relationship between time and active enzyme concentration. Sadana (1991) proposed the concept of "grace period". The grace period is time during which the enzyme does not lose any of its activity. The time delay phenomenon is common in living systems, for instance, it is known that there is time delay between the introduction of living cells to a new growth environment and their actual growth. A time delay model for enzyme degradation can be formulated as:

$$\frac{E}{E_0} = 1 \qquad \ldots \text{for } t \le t_d \qquad (6)$$

And Equation 5 applies for $t > t_d$, where t_d is the delay time and E_0 is the initial enzyme concentration (activity) at time zero. This model requires the determination of k_d and t_d.

As early as 1950, Schwimmer reported the inhibition of amylase hydrolysis by dextrins and maltose. It was found that maltose inhibits the hydrolysis process in a non-competitive manner over a wide concentration range. Limit dextrin inhibited hydrolysis in a non-competitive manner while α-dextrin demonstrated competitive inhibition. Weselake and Hill (1983) reported that maltose was a non-competitive inhibitor but Houng *et al.* (1992) found maltose inhibition to be competitive. Akerburg (2000) reported that glucose inhibited wheat starch saccharification. Hill *et al.* (1997) presented an empirical model for amylase inhibition during starch particle hydrolysis by both maltose and glucose. The model is a simple exponential inhibition function:

$$\frac{dS}{dt} = \left(\frac{dS}{dt}\right)_{P=0} \times \exp(-k_i * P) \qquad (7)$$

Substituting the time delay model (Equations 5 and 6) and the exponential inhibition model (Equation 7) into the prediction of product concentration (Equation 3) gives the following two expressions for predicting the dynamics of product formation from the hydrolysis of starch particles:

$$\frac{dP}{dt} = \frac{K(S_0 - P)E_0 \exp(-k_i P)}{K_A + E_0} \quad \ldots \text{for } t \le t_d \qquad (8)$$

$$\frac{dP}{dt} = \frac{K(S_0 - P)E_0 \exp(-k_i P)\exp(-k_d (t - t_d))}{K_A + E_0} \quad \ldots \text{for } t > t_d \qquad (9)$$

Materials and methods

Chemicals:

Barley α-amylase (catalogue # A-2771), 3-aminopropyl-triethoxysilane (APES) and silica particles with diameters between 0.5 and 10 μm (catalogue # S-5631) were purchased from Sigma. Glutaraldehyde (50% v/v) was used to activate the silanized carrier particles and was purchased from VWR Scientific. All immobilization experiments were carried out in 50 ml sterilized centrifuge tubes made of polypropylene (VWR catalogue # 21008-690).

Total protein concentrations of the immobilizing solution (buffer solution containing barley α-amylase) before and after the enzyme-coupling step (incubation of activated carrier particles in enzyme solution) were determined to see if the enzyme had been immobilized onto the surface of the carrier particles. The chemicals used for this determination were: Coomassie Blue G250 from Sigma and 85% phosphoric acid and 95% ethanol supplied by VWR Scientific. The difference between the amount of total protein in the supernatant of the immobilization solution before and after the enzyme-coupling step indicated how much enzyme had been immobilized onto the carrier particles.

The enzymatic activities of barley α-amylase, both soluble and immobilized, were determined based on the method suggested by Miller (1959). Soluble starch and sodium hydroxide were purchased from Fisher Scientific. 3,5-dinitrosalicylic acid and sodium potassium tartrate were supplied by Sigma. Sodium phosphate monobasic was supplied by BDH.

The starch used in the cold starch hydrolysis study with immobilized barley α-amylase was Wheat Starch 4 donated by CSP Mills, Saskatoon, but manufactured by Archer-Daniels-Midland/Ogilvie in Montreal. Wheat Starch 4 is a class of A grade starch which ranges from 18 – 20 μm (manufacturer's specifications) in size.

Immobilization of enzyme:

The method of enzyme immobilization was adapted from that suggested by Weetall and Filbert (1974) and Wiseman (1995). For the silanization process, 3 g of carrier support particles were incubated in 50 ml of acetone containing 10 % APES. The suspension was incubated in a centrifuge tube at 200 revolutions per minute (rpm) at 23 °C for about 17 hours. The treated carrier support particles were then centrifuged (3,500 rpm for 5 minutes), decanted and dried in a vacuum oven at 75 °C and -80 kPa for 30 minutes before the dried particles were rinsed three times with deionized water. Every washing was followed by centrifugation at 3,500 rpm for 5 minutes.

Two methods were investigated for glutaraldehyde activation, simultaneous and sequential. For sequential activation, each preparation of silanized precipitate (approximately 3 g) was activated with 30 ml of 2.5 % glutaraldehyde solution in 0.1 M sodium phosphate monobasic at pH 6.5. The preparation was incubated either at 23 °C or 4 °C, 200 rpm for one hour. The activated preparation was washed with deionized water and centrifuged (3,500 rpm for 5 minutes) three times to wash off the residual glutaraldehyde that might contaminate the activated preparation. During the enzyme-coupling stage, the activated preparation was incubated with enzyme solution as described below.

For simultaneous activation, the silanized particles were incubated in 2.5 % glutaraldehyde solution in 0.1 M phosphate buffer at pH 7.0, 30 mg enzyme/ml, at 200 rpm for one hour. The final preparation for this option was washed with deionized water and centrifuged (3,500 rpm for 5 minutes) three times to wash off the residual glutaraldehyde and enzyme in the solution.

The activated support for the sequential immobilization process was subsequently coupled to barley α-amylase. Barley α-amylase at different concentrations (to be optimized) was dissolved in 0.1 M phosphate buffer at pH 7.3. The enzyme solution was tested for its total protein concentration. The activated preparation (approximately 3 g) was incubated in 10 ml of the barley α-amylase solution at either 4 ^{o}C or 23 ^{o}C (to be optimized) at 200 rpm for 12 hours. The preparation was then centrifuged at 3,500 rpm for 5 minutes. The supernatant of the preparation was tested for its total protein content. The final (enzyme coupled) particles were centrifuged and rinsed alternately with deionized water three times. The supernatant of the preparation after the third centrifugation was tested for the presence of protein to make sure that the enzyme coupled preparation was not releasing residual enzyme into solution. The enzyme coupled preparation was either re-suspended in buffer solution or left to dry at room conditions. The total protein concentrations measured in the immobilization experiments were not further utilized in calculations of the specific activity of the immobilized enzyme. The difference between the total protein concentrations before and after the enzyme coupling incubation step was used only as an indicator that enzyme had been immobilized onto the carrier support particles.

This was because the enzyme source was not highly purified and protein measurement could not therefore be used as a quantitative measurement of α-amylase.

Inhibition experiments:

Starch granules at 100 g/L and maltose or glucose (both analytic grade from VWR Scientific) at different concentrations were prepared in sterile reaction media in well-mixed reactors (similar in design to those reported earlier by Textor et al., 1998). The reactors were subsequently brought to the desired reaction temperature in an environmental chamber. After ten minutes of stirring at 400 rpm (stirring speed which resulted in completely well-mixed solution), the immobilized α-amylase suspension was introduced into the reactors that were sampled for about ten hours. The samples were measured for turbidity as an indication of starch concentration.

Thermostability experiments:

Immobilized barley α-amylase prepared during the immobilization stage was re-suspended in 0.02 M sodium acetate and 0.002 M calcium chloride solution and was then poured into the kinetic reactor without the presence of wheat starch. The immobilized enzyme suspension was stirred at 400 rpm at temperatures of 20, 30, 45 and 55 °C in an environmental chamber. Samples were taken at various time intervals for enzyme activity assays.

Starch granule hydrolysis experiments:

Freshly prepared, immobilized barley α-amylase was re-suspended in the reaction medium (0.02 M sodium acetate and calcium chloride between 0.001 and 0.004 M) just before starting the experiments. Wheat starch solution of specified concentrations was

prepared in bulk and pipetted into the kinetic reactors followed by the immobilized enzyme suspension. The final working volume in each reactor was 25 ml. The reactors were then immediately placed in the environmental chamber and either turbidity or chemical oxygen demand (COD) was monitored. For turbidity analysis, 60 to 120 μl of sample was diluted to 3 ml with deionized water before the absorbance of the sample was measured at 600 nm. For COD analysis, 500 μl of sample was centrifuged at 11,000 rpm for 10 minutes before 50 to 100 μl of the supernatant was pipetted into a vial for dilution to 3 ml with deionized water. The diluted sample was tested for COD, maltose and glucose concentrations.

Procedures for long-term (operational) stability experiments were essentially the same as those described in the preceding paragraph except that each kinetic reactor was sampled only one day, demanding multiple reactors for each run.

Analysis:

Enzyme activity is expressed in two ways in this investigation. The enzyme immobilization experiments were quantified in terms of units per gram of silica, where one unit is equivalent to the production of one milligram of maltose produced in three minutes from the hydrolysis of soluble starch. For hydrolysis of starch particles, the enzyme concentration is expressed in terms of equivalent soluble enzyme per milliliter. This concentration was calculated from knowledge of the activity of the immobilized silica particles (in units per gram of silica), the quantity of active silica added to the reaction vessels and the fact that one milligram of the soluble enzyme powder was found to have an activity of 0.108 units when used to hydrolyze soluble starch.

Total protein concentration, COD, enzyme activity and turbidity of the reaction solution were measured using a spectrophotometer (model LKB Novaspec II). Total protein concentrations of the enzyme solutions were determined by the Bradford method (Ninfa and Ballou, 1998). Barley α-amylase activity was measured based on the assay suggested by Miller (1959).

The turbidity of the reaction solution was measured at 600 nm to monitor the degree of solubilization of wheat starch granules in the reaction medium. Due to the presence of the immobilized enzyme, the concentration of wheat starch granules could not be determined quantitatively through turbidity analysis. However, measurement of the turbidity of the reaction solution provided qualitative information about the solubilization of the wheat starch granules. Reference experiments confirmed that the turbidity of the reaction medium did not change when wheat starch granules were incubated with silica particles and no enzyme. Therefore, it was concluded that the decrease in turbidity in the reaction medium was caused by wheat starch solubilization. Turbidity analyses were performed to study the effects of inhibition, pH, temperature and calcium concentration.

The COD was determined using a Hach model 45600 COD reactor. The measurement of dissolved carbon was conducted by oxidizing the sample in reagent vials (Hach catalogue #21259-15) at high temperatures.

Maltose and glucose concentrations were measured using a Sugar Pak I column (Waters Corporation, Milford, Mass.) in a Hewlett Packard Series 1100 high performance liquid chromatograph with a refractive index detector. The mobile phase was 50 ppm calcium EDTA in deionized water that was maintained at 80 °C and flowed at 0.5 ml/min.

Results and discussion

Immobilization optimization:

Simultaneous vs Sequential: Glutaraldehyde is widely used for cross-linking proteins onto solid matrices (Walt and Agayn, 1994). The question of whether the glutaraldehyde activation step should be performed simultaneously or sequentially is a critical issue. For instance, Nazari (1998) immobilized glucose oxidase and catalase on nylon membranes via the covalent binding method. It was found that the simultaneous strategy gave greater glucose oxidase activity while the sequential method yielded greater catalase activity. In these experiments, for the simultaneous immobilization strategy, the activity of barley α-amylase was found to be 0.15 ± 0.01 unit/g of silica, while that of sequential immobilization was 0.46 ± 0.06 unit/g. During simultaneous immobilization, inter-molecular cross-linking yielded aggregates of the enzyme, reducing the effective concentration of active free enzyme in the solution. Sequential addition of glutaraldehyde and enzyme, however, prevented cross-linking of the enzyme and consequently the activity was significantly higher. Therefore, sequential immobilization was used for the rest of the experiments.

Temperature: It has been recommended that the glutaraldehyde activation step should be carried out at room temperature while the enzyme-coupling step should be at 4 °C (Weetal and Filbert, 1974; Nazari, 1998). Table 1 compares the activities of the barley α-amylase immobilized under different temperature conditions. It was found that the immobilized enzyme was almost equally active, regardless of the glutaraldehyde activation and enzyme

coupling temperatures. Consequently, the two steps of the experiment were performed at 23 °C in all subsequent experiments.

Enzyme concentration: The optimal concentration of barley α-amylase in the immobilizing solution to produce the most active immobilized enzyme was then investigated. In each experiment, 3 grams of silica particles were immersed in 10 ml of immobilizing solution. At concentrations higher than 2.0 g of enzyme (200 mg/ml), the immobilizing solution became over-saturated with enzyme, i.e. further dissolution of the enzyme powder in the solution was not possible (precipitate in the solution). It is apparent in Figure 1 that maximum immobilized enzyme activity (11.5 ± 0.3 unit/g) was achieved at an enzyme concentration of 1 g (100 mg/ml).

Calcium ion effects: α-amylases are metallo-enzymes. Calcium helps to stabilize the tertiary structures of these enzymes. Table 2 shows the activities of barley α-amylase immobilized in the presence of calcium ion at various concentrations. The highest activity was 13.7 unit/g silica for samples incubated in 0.005 M Ca^{2+} in deionized water. However, at the 70 % confidence limit, there was no statistical difference between the measured activities at calcium ion concentrations up to 0.05 M. At higher calcium ion concentrations, the resulting activity decreased. Kvesitadze and Dvali (1982) reported that calcium ion significantly enhanced the immobilized bacterial α-amylase activity at concentrations up to 0.5 M. However, Sogaard et al. (1999) recently reported that the activity of barley α-amylase decreases above 0.05 M and suggested that this is due to extra calcium binding sites which inactivate the active sites on the enzyme molecule.

Storage of immobilized enzyme: The effect of storage conditions on the activity of the immobilized enzyme is an important aspect to ensure that a long shelf life is possible. The decline of enzyme activity was essentially identical for all storage conditions tested. Barley α-amylase lost its activity at a rate of 10 % for every 12 days of storage over a period of 30 days, whether it was stored at 4 °C or 23 °C, wet or dry. This degradation rate was similar to the case of soluble barley α-amylase, except that the activity profile of the soluble enzyme at 4 °C with the presence of calcium was observed to increase to its initial activity level after about the 17th day of storage. It then began to lose activity once again at a rate of 10 % every 12 days. This phenomenon was confirmed by performing the storage experiment with soluble barley α-amylase twice. Roig *et al.* (1993) reported a similar increase in the activity profile for a soluble α-amylase from a bacterial strain.

Thermostability:

Figure 2 depicts enzyme thermostability profiles for five sets of experiments carried out at different temperatures. The deactivation patterns of the immobilized enzyme at 20, 30 and 45 °C clearly shows a pure time delay before the activity of the enzyme begins to degrade. During this time delay, the enzyme activity slightly increased above its value at time zero, perhaps due to continued incubation of the immobilized enzyme complex which increased the availability of active sites. At 55 °C no time delay was observed and the immobilized enzyme activity decreased very quickly. The enzyme time delay model (Equations 5 and 6, lines in Figure 2) was found to fit the deactivation pattern of both soluble and immobilized barley α-amylase well. The best fit values of the time delay (t_d) and degradation constant (k_d) are listed in Table 3. It is easy to observe that lower

temperatures increase the time delay and decrease the rate at which the enzyme activity degrades. It can also be concluded from Figure 2 that immobilization has significantly increased the thermostability of barley α-amylase. At 45 °C, the immobilized enzyme had a time delay three times longer and degraded with a rate constant three times lower than the soluble enzyme. Clearly the immobilization process has enhanced the thermostability of barley α-amylase. The Arrhenius plot of the first order degradation rate constant for immobilized barley α-amylase is shown in Figure 3. The rate constant follows the Arrhenius relationship well and results in an activation energy of enzyme degradation of 66.4 kJ/mol.

Hydrolysis of starch granules using immobilized barley α-amylase:

Enzyme-catalyzed reactions, like other chemical reactions, increase in rate with temperature (Wiseman, 1995). Textor et al. (1998) found that soluble barley α-amylase had higher activity (in terms of wheat starch solubilization rate) at higher temperatures, but the enzyme also degrades faster as the temperature increases. The optimal temperature for starch granule hydrolysis using soluble barley α-amylase was 45 °C. Figure 4 shows the relative turbidity profiles of the reaction solutions using immobilized barley α-amylase at three temperatures. The activity of the enzyme was similar at 40 °C and 45 °C, both of which were significantly higher than at 30 °C. However, lower temperatures (30 °C) are more desirable for the hydrolysis processes in terms of conservation of energy and would be necessary for simultaneous hydrolysis and fermentation.

Again using turbidity analysis, and similar to the findings of Textor *et al.* (1998) who used soluble barley α-amylase, the pH optima for wheat starch granule hydrolysis by

immobilized barley α-amylase was found to be 4.5. At pH values of 4.0 and 7.0, the hydrolysis rate was very low. The optimal calcium ion concentration was found to be 0.002 M in terms of the final relative turbidity values. This observation agrees with Lang et al. (2001a).

Figure 5 shows the inhibition of the immobilized hydrolysis reaction at maltose and glucose concentrations up to 250 g/L. The exponential inhibition model proposed earlier by Hill *et al.* (1997, Equation 7) fit the data with less than 7 % deviation (solid line shown in Figure 5). The best-fit value of the inhibition parameter (k_i in Equation 7) was found to be 0.0073 ml/mg. Hill *et al.* (1997) postulated that one possible reason for this type of inhibitory pattern is the reduced water activity in the vicinity of the enzyme-starch complex. Also shown on Figure 5 is the inhibition model of Hill et al. (1997) using soluble barley α-amylase (dashed line). It can be observed that the immobilization process has not significantly stabilized the enzyme from inhibition by the sugar product molecules.

Figure 6 shows the dynamic hydrolysis of wheat starch granules using immobilized barley α-amylase at a concentration of 30 mg/ml, 40 °C, pH 4.5 and using 0.002 M calcium chloride. 30 mg/ml of enzyme was found to produce optimum conversion of the starch granules at fast hydrolysis rates, similar to the findings reported earlier by Textor et al. (1998) but using soluble barley α-amylase. In Figure 6, the conversions are shown as soluble COD. Reference reactors containing only immobilized enzyme and reactors containing starch alone showed no evidence of soluble COD, so that the COD data in Figure 6 is due solely to starch granule hydrolysis. It can be observed that at 30 mg/ml, the immobilized barley α-amylase was able to hydrolyze starch granules at concentrations as

high as 100 mg/ml. The final conversions after 38 hours of hydrolysis were 100 % for 32 and 60 mg starch granules/ml, 94% for 75 mg starch granules/ml and 90% for 100 mg starch granules/ml. It can also be observed from Figure 6 that more than 60% of the starch granules have been hydrolyzed after the first 8 hours even for runs with high starch granule concentrations.

The immobilized enzyme was tested for its long-term, operational stability by repeatedly using the enzyme in fed-batch mode to hydrolyze wheat starch granules. Lang et al. (2001a, 2001b) found that the fed-batch reaction mode was superior to simple batch and continuous operations using soluble barley α-amylase. To achieve fed-batch conditions, the contents of each reactor were centrifuged, the supernatant was decanted off, and fresh solution containing starch granules were recharged into the reactors after every 24 hour period to return the starch concentrations to 100 mg/ml. The initial COD concentrations following day one are not zero due to carry-over of some product with the precipitate from the previous day's run. Two sets of experiments were completed, one at 30 °C and one at 40 °C, using an enzyme loading of 30 mg/ml and a calcium chloride concentration of 0.002 M. Figure 7 shows hydrolysis performance at 30 °C, which was very similar to the observations made at 40 °C except that the starch conversion was slightly faster at 40 °C and remained that way for four days of operation. The extent of starch conversion was 83 % for day one, 53 % for day two, 25 % for day three and averaged about 20 % on day four, after about 100 hours of operation. The 40 °C run was performed in duplicate and had a somewhat greater extent of hydrolysis on the fourth day of operation compared to the 30 °C run, but continued to degrade such that no activity was

observed on the ninth day (data not shown). Lang et al. (2001a) reported a very similar loss of activity with soluble barley α-amylase. It appears that the immobilization process developed here has not significantly improved the operational stability of barley α-amylase.

As can be seen by the solid lines in Figures 6 and 7, good agreement between the experimental data and the hypothesized model (Equations 8 and 9) was obtained for hydrolysis of starch granules by immobilized barley α-amylase. The model includes the effects of both product inhibition and enzyme degradation. The best-fit values of the reaction constants in the model are listed in Table 4. The enzyme activity decayed at a rate four times faster than had occurred during thermostability studies at 30 °C, as can be seen by comparing the degradation constants in Tables 3 and 4. Unlike the thermostability studies, the degradation rate of the enzyme was the same for both 30 and 40 °C, indicating that the operational stability of the immobilized enzyme was controlling the decline in hydrolysis performance. Similar to this study, Lang et al. (2001a) found that the soluble enzyme could only be used for four sequential hydrolysis runs using an ultrafilter to keep the soluble enzyme in the reactor. The immobilization process developed here seemed to only reduce the degradation rate of the enzyme during thermostability studies, but once hydrolysis was taking place, deactivation proceeded at the same speed as for the soluble enzyme molecules.

The hydrolysate from cold wheat starch hydrolysis was further analyzed using HPLC. It was discovered that the soluble sugars were made up of about 60 % maltose and 40 % higher molecular weight dextrins. No glucose was detected in the product solutions. These results are in agreement with Zackova and Kvasnicka (1994) who studied the

hydrolysis of wheat starch by wheat malt. Those authors found that maltose made up the largest amount of sugar in their hydrolysates (65.3%) while the glucose concentration was very low (2.6%).

Conclusions

Barley α-amylase has been successfully immobilized onto silica particles using the covalent binding method. The binding method consists of three steps: APES treatment, glutaraldehyde activation and enzyme coupling. The immobilized barley α-amylase was found to be more stable (in terms of thermostability) than its soluble counterpart.

The immobilized barley α-amylase is able to hydrolyze wheat starch granules at temperature below the gelatinization temperature. The optimal condition for cold starch hydrolysis using immobilized barley α-amylase is: 40 °C, pH 4.5, 0.002 M calcium ion in the reaction medium and an immobilized enzyme loading of 30 mg/ml. The immobilized enzyme is able to hydrolyze starch granules up to 100 mg starch granule/ml with a final conversion of about 90 %, which can be achieved in about 24 hours. Over 60 % of the starch is hydrolyzed after the first eight hours of operation. The immobilized enzyme was inhibited by maltose and glucose similar to the case of the soluble enzyme. An exponential model was shown to describe the inhibitory effect of maltose and glucose.

A kinetic model developed for soluble-enzyme, insoluble-substrate system has been found to describe the reaction kinetics of immobilized-enzyme, insoluble-substrate well. The model predicted the effects of starch granule concentration, enzyme degradation and repeated fed-batch hydrolysis runs extremely well. The immobilized process did not

enhance the operational stability of barley α-amylase, which declined after four sequential uses, or about 100 hours of continuous, fed-batch operation.

Recommendations

The idea of immobilizing barley α-amylase on small silica particles has been shown to be possible using a commercial, crude mixture of this protein. The immobilized enzyme was shown to be capable of hydrolyzing starch granules at the same rates and as effectively as soluble barley α-amylase. The immobilized enzyme was three times more thermally stable than the soluble enzyme, but decayed at the same rate as the soluble enzyme during the hydrolysis of starch granules. It would now be useful to investigate whether an environmental or chemical conditioning method can be developed that would make the immobilized enzyme stable during hydrolysis operations. If this could be achieved, a very useful, new economical product would have been developed.

Based on the results of the previous study using soluble barley α-amylase, it is recommended that a pilot-scaled soluble barley α-amylase bioreactor be built. This is required to show that the experimental process can be successfully carried out at larger scales. This would be the last step required prior to building a full-scale process, which has already been predicted to be incredibly profitable.

Nomenclature

0	initial condition subscript
A	adsorption site concentration (sites/ml)
E	enzyme concentration (mg/ml or units/mg)
K	rate constant in Equation 2 (ml/mg-h)
K_A	saturation constant in Equation 2 (mg/ml)
k_d	degradation rate constant in Equation 4 (h^{-1})
k_i	product inhibition constant in Equation 7 (ml/mg)
P	product concentration (mg/ml)
S	starch concentration (mg/ml)
t	time (h)
t_d	delay time (h)

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Table 1. Immobilization Temperature Effects on Barley α-Amylase Activity

Activation Temperature APES / Glutaraldehyde	Immobilization Temperatue Barley α-Amylase	Activity* (unit / g silica)
4 °C	4 °C	9.6 ± 1.0
4 °C	23 °C	9.2 ± 0.5
23 °C	4 °C	9.8 ± 1.9
23 °C	23 °C	10.7 ± 0.1

*results are averages of three measurements at each condition

Table 2. Calcium Ion Effects on Barley α-Amylase Activity

Solution	Calcium Concentration (M)	Activity* (unit / g silica)
water	0	12.1 ± 1.5
buffer	0	11.7 ± 1.5
water	0.005	13.7 ± 2.8
water	0.05	11.7 ± 1.6
water	0.2	8.8 ± 2.2
water	0.5	6.6 ± 0.9

*results are averages of three measurements at each condition

Table 3. Best Fit Deactivation Rate Constants for Immobilized Barley α-Amylase (pH 4.5, 0.002 M calcium ion)

Temperature (°C)	t_d (h)	k_d (h^{-1})
55 (immobilized)	0	0.089
45 (immobilized)	8.5	0.028
45 (soluble)	2.8	0.097
30 (immobilized)	23	0.013
20 (immobilized)	67	0.0040

Table 4. Best-fit Parameters in Equation 8 and 9 for Hydrolysis of Starch Granules Using Immobilized Barley α-Amylase (pH 4.5, 0.002 M calcium ion)

Temperature (°C)	K (ml/mg·h)	K_A (mg/ml)	k_d (h^{-1})
30	280	1.9e05	0.055
40	500	1.9e05	0.055

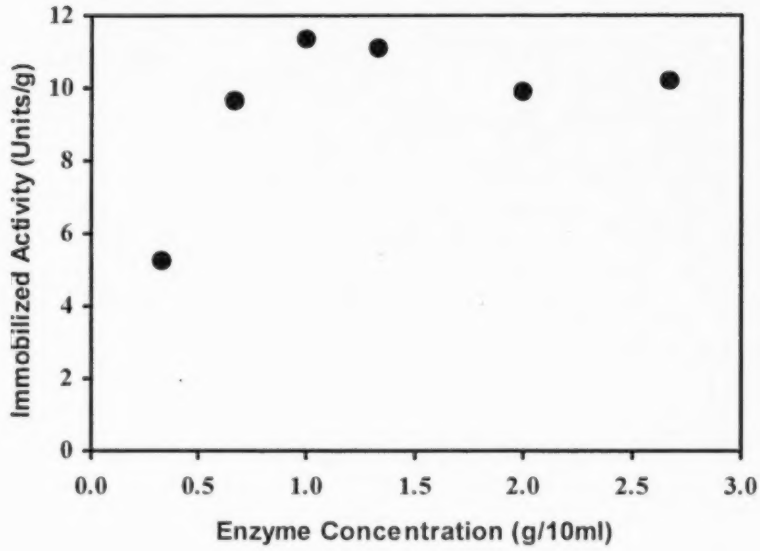


Figure 1. Effect of enzyme concentration during the immobilization coupling stage on the activity of immobilized silica particles (concentration of silica was 0.3 g/ml, 23 °C).

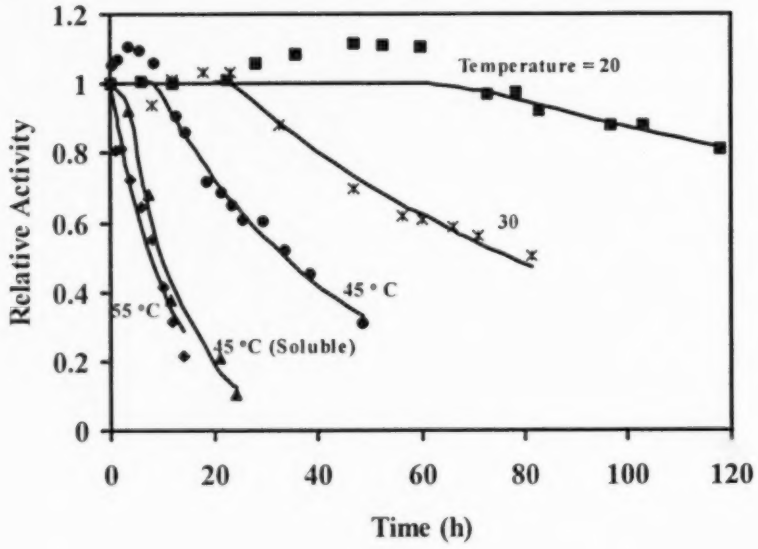


Figure 2. Thermostability profiles of immobilized barley α-amylase held in 0.02 M sodium acetate and 0.002 M calcium chloride solution in a well mixed vessel.

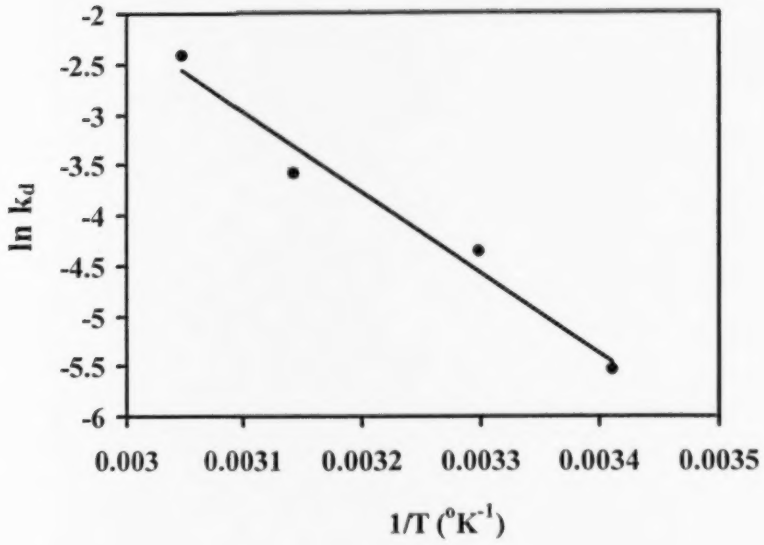


Figure 3. Arrhenius plot showing the effect of elevated temperatures on the thermal decay of barley α-amylase.

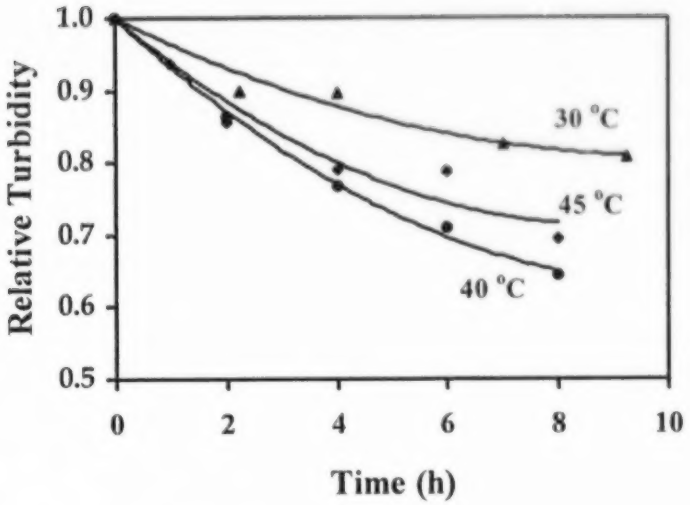


Figure 4. Effect of temperature on the initial rates of starch granule hydrolysis by immobilized barley α-amylase (100 mg/ml of granules, 30 mg/ml of immobilized enzyme, 0.002 M calcium chloride, pH 4.5).

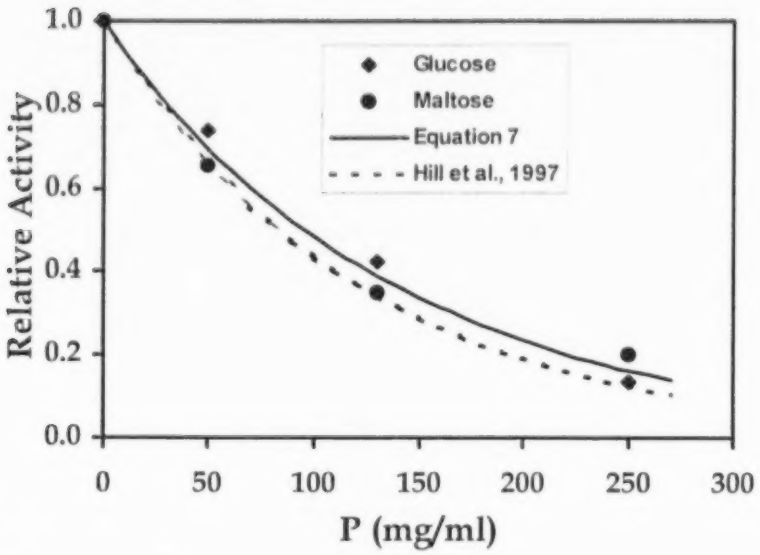


Figure 5. Maltose and glucose inhibition of starch granule hydrolysis by immobilized barley α-amylase (100 mg/ml of granules, 30 mg/ml of immobilized enzyme, 0.002 M calcium chloride, pH 4.5, 40 °C).

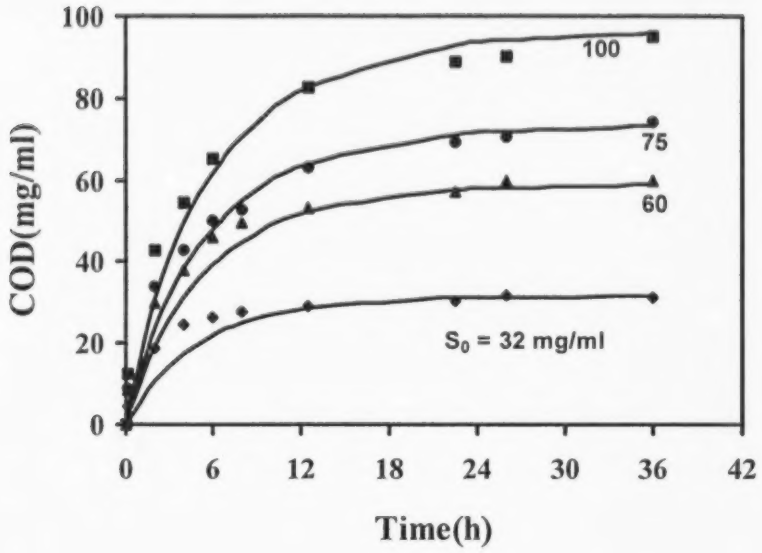


Figure 6. Product formation dynamics for the hydrolysis of starch granules by immobilized barley α-amylase (30 mg/ml of immobilized enzyme, 0.002 M calcium chloride, pH 4.5, 40 °C).

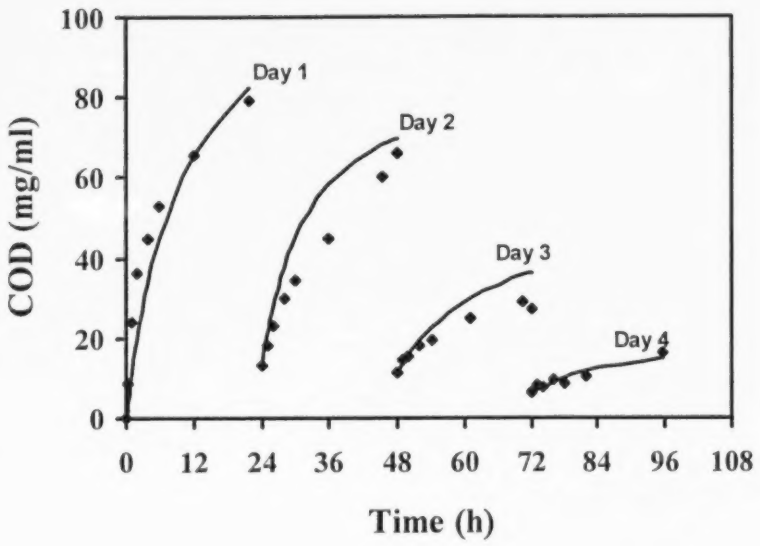


Figure 7. Repeated fed-batch hydrolysis of starch granules by immobilized barley α-amylase at 30 °C (100 mg/ml of granules at start of each day, 30 mg/ml enzyme at start of day one only, 0.002 M calcium chloride, pH 4.5).

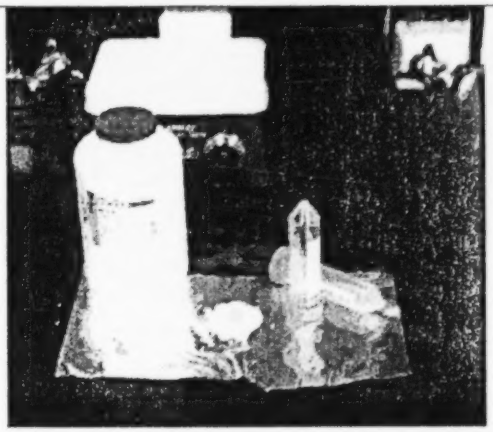
E. Equipment

Nil.

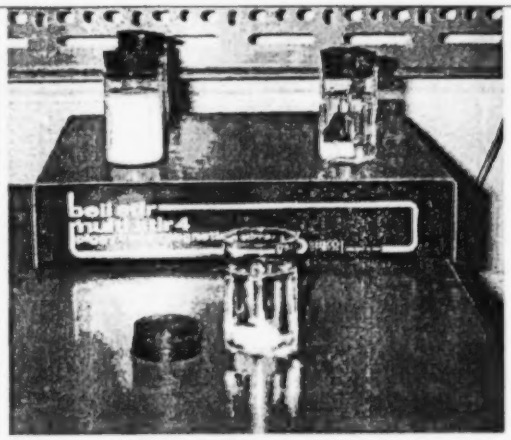
F. Project Developed Materials

- This report.
- A preliminary Powerpoint presentation of this study was presented to the Department of Chemical Engineering in March 2001 and will be revised and presented at the 51st Canadian Chemical Engineering Conference in October, 2001.
- A journal manuscript (in preparation).
- Calvin Lim's MSc (ChE) Thesis.

G. Project Photos



Picture of Silica Particles and Tubes Used for Incubation Enzyme with Particles.



Picture of Glass 25 ml Reactors Used for Hydrolysis Experiments.

H. Acknowledgements

The Saskatchewan Department of Agriculture and Food has been acknowledged in all our presentations and publications (see attached journal publications). This will be continued in our Halifax presentation and in the manuscript under preparation.